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Bio-statistical evaluation of cultural conditions on industrial textile dye decolourisation using a native bacterium *Micrococcus endophyticus* (ES37)

P. M. Ayyasamy, Suresh S. S. Raja, B. Subashni and R. Palanivelan

ABSTRACT

An indigenous dye-decolourising bacterium *Micrococcus endophyticus* (ES37) was isolated from dye contaminated soil and identified by 16S rDNA sequencing. The bacterial strain ES37 exhibited 97.19% of dye removal capacity in Luria-Bertani broth composition within 48 h, while the culture containing yeast extract showed 53.4% decolourisation in 72 h. In the absence of carbon and nitrogen sources, the bacterial strain failed to decolourise the dye, even on extended incubation. The effect of environmental factors on decolourisation was investigated by Plackett–Burman design and the significant parameters were lactose, yeast extract and pH. Optimisation of these factors was done by response surface methodology with central composite design; the decolourisation ranged from 0.43 to 77.49%. The optimised levels of lactose, yeast extract and pH were found to be 0.85% (w/v), 0.71% (w/v) and 7.5%, respectively. Under the optimal conditions, decolourisation of remazol golden yellow by ES37 strain was 81.61%, which was in agreement with the predicted value of 79.99%. These findings revealed the interactions and importance of environmental factors on dye decolourisation using native bacteria and also their standard point for an effective dye removal process.

Key words | azo dyes, environmental parameters, *Micrococcus endophyticus*, remazol golden yellow, textile dye decolourisation

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ABBREVIATIONS

ANOVA	analysis of variance
BOD	biological oxygen demand
CCD	central composite design
COD	chemical oxygen demand
DOE	design of experiments
PBD	Plackett–Burman design
PCR	polymerase chain reaction
RSM	response surface methodology

INTRODUCTION

Nowadays, management of water pollution is one of the major environmental challenges for ecologists due to rapid industrialisation. Enormous amounts of chemicals including dyes are manufactured and utilised in materials, such as textiles, printing, pharmaceuticals, foods, toys, papers, plastics and cosmetics (Moosvi *et al.* 2007). Among these, discharge of coloured effluents from dye manufacturing units and textile processing industries impose the majority of environmental problems. The effluents from these dyeing industries increase the biological oxygen demand (BOD) and chemical oxygen demand (COD) levels in water bodies. The quantity and composition of effluents depend upon the dyeing process and the type of dye being used. Among all dyes, the azo group of dyes comprises about 70% occurrence in effluents due to its wide range of application in textile industries (Khouni *et al.* 2012).

Prior to dyeing, grey fabrics are consigned to washing, scoring, desizing and bleaching practices (Aly *et al.* 2010). In textile industries, high concentrations of salt are utilised for fixation of

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fibre reactive dyes on fabric materials. Textile wastewater also contain heavy metals, which are combined with the azo dye complex in order to resist biological degradation; this causes serious health hazards when it comes in contact with domestic water (Cooper 1995). Draining of coloured effluents into an aquatic environment generates a foul smell, reduces oxygen levels, sunlight penetration and pH, which in turn affects the natural ecosystem (Asgher et al. 2009; Molina-Guijarro et al. 2009). Enormous quantities of textile wastewater also pollute groundwater and soil fertility (Pourbabaee et al. 2005).

The dyes that are released into the water bodies are carcinogenic and mutagenic in nature and are hoarded in living organisms through bioaccumulation (Lima et al. 2007). For instance, the presence of monoazo and anthoquinone groups in dyes are found to cause allergic reactions (Vander Zee et al. 2003). In the human body, the liver and kidneys metabolise azo dye, using the azo reductase enzyme, into aromatic amines; this is then excreted in the urine. Sometimes, it causes bladder cancer and induces tumour formation (Brown & Devito 1993).

Currently, various physical, chemical and biological treatment technologies have been implemented for removal of colour from the effluents of textile dyeing industries (Mahmoued 2010). The main drawbacks in the implementation of conventional physical and chemical methods are the high cost, low efficiency, inapplicability to a wide variety of dyes and also the generation of secondary wastes (Rauf & Salman Ashraf 2009). Microbial decolourisation is an eco-friendly and cost-effective method that produces less sludge compared to chemical decomposition processes (Verma & Madamwar 2003).

In the last few decades, much research has focussed on biodegradation or bioaccumulation of dyes using autochthonous micro-organisms, due to their ability to survive in higher pH, temperature, salt concentration and a nutrient deficient medium. Therefore, biological treatment of dye removal, involving various operational parameters, must be analysed and optimised prior to on-site execution. Conventional optimisation methods have resulted in a large number of unreliable experiments at an incomplete stage (Senthilkumar et al. 2012).

Statistical tools, such as design of experiments (DOEs), have assisted in predicting the optimum response value with a few experiments (Montgomery 2001). Plackett–Burman design (PBD) and response surface methodology (RSM) application are used to find combined and individual effects of the significant factors that are involved in the process (Plackett & Burman 1946; Chen 1994). Mostly, they are used to reduce the chemical usage and the time involved in the process by optimisation (Ayed et al. 2011). Optimisation of dye decolourisation has specifically focussed on the factors playing an important role in large-scale treatment, by a bio-statistical approach using bacteria that was not explored well in dye decolourisation.

The present study aimed to explore the potentiality of native bacteria (which were isolated from dye contaminated soil) in the dye decolourisation process, and also to employ them in decolourisation of a reactive azo dye, remazol golden yellow.

MATERIALS AND METHODS

Isolation and screening of dye-decolourising bacteria

Dye contaminated soil was collected in sterile polyethylene bags from a textile industry located in Erode, Tamil Nadu, India. For bacterial isolation, 1.0 g of soil sample was subjected to dilution and cultured using a pour plate technique in nutrient agar plates containing 5 g/L peptone, 5 g/L sodium chloride, 3 g/L yeast extract, 20 g/L agar (pH 7.0 ± 0.2). Inoculated plates were incubated at 35 ± 2 °C and 45 ± 2 °C for 24–48 h in order to isolate mesophilic and thermophilic bacteria, respectively. All steps in the culture isolation and inoculation procedure were carried out in a sterile cabinet under laminar flow conditions. The distinct bacterial colonies were purified by repeated streaking on nutrient agar; their dye decolourisation ability was then tested. The dye decolourisation proficiency of refined bacterial isolates was tested by dot plate assay in Luria-Bertani agar plates containing 10 g/L casein enzymic hydrolysate, 5 g/L yeast extract, 10 g/L sodium chloride and 15 g/L agar amended with various concentrations (50, 100, 150, 200 and 250 mg/L) of remazol golden yellow dye (RNL). The inoculated plates were incubated at 37 °C for 4 days and the decolourising zones around the bacterial colonies were observed.

Identification of dye-decolourising bacteria

The selected feasible dye-decolourising bacterial strain ES37 was identified by the 16S rDNA sequencing method. The

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genomic DNA isolation, extraction, polymerase chain reaction amplification and 16S rDNA sequencing of the amplified gene were carried out at Xcelris Labs Ltd, Ahmedabad, India.

Phylogenic tree analysis and Genbank accession

The generated 16S rDNA consensus sequences were initially analysed in an NCBI server (www.ncbi.nlm.nih.gov/BLAST) using the BLASTN tool. Closely related sequences were obtained and aligned with the multiple alignment software program Clustal W. A phylogenetic tree was constructed by the neighbour joining (NJ) method in MEGA version 5.0 based on bootstrap analysis (1,000 replicates). The nucleotide sequences were deposited in the NCBI GenBank with accession number JX042488.

Decolourisation efficiency in various media

The decolourisation competence of selective bacterial strains was determined with various aqueous media containing a 100 mg/L concentration of remazol golden yellow dye, namely: (i) Luria-Bertani broth: 10 g/L casein enzymic hydrolysate, 5 g/L yeast extract, 10 g/L sodium chloride; (ii) yeast extract broth: 5 g/L yeast extract and 5 g/L sodium chloride; (iii) Bushnell and Hass broth: 0.2 g/L magnesium sulphate, 1 g/L di-potassium hydrogen phosphate, 0.02 g/L calcium chloride, 0.05 g/L ferric chloride, 1 g/L ammonium nitrate. About 1% (v/v) of 24 h culture was inoculated and incubated at 37 °C for 3 days, under static condition. Every 24 h, the samples were withdrawn and centrifuged at 5,590 × g for 20 min. Absorbance (OD) of the cell free supernatant was measured using UV spectrophotometer (Cyberlab UV-100, USA) at 412 nm. Experiments were conducted in triplicate; the mean values were used to determine the decolourisation percentage using Equation (1) as follows:

$$\text{Decolorisation}\ (\%) = \frac{\text{Initial absorbance value} - \text{Final absorbance value}}{\text{Initial absorbance value}} \times 100 \tag{1}$$

Optimisation of dye decolourisation via DOEs

DOEs is a bio-statistical tool, which reveals the individual and interactive effects of multiple factors involved in a process, by screening up to 'n–1' variables in just 'n' number of trials. Estimation of regression coefficients and prediction of the fittest model were carried using the statistical software Minitab Version 15 (Tripathi & Srivastava 2012).

Plackett–Burman design

The 2 k-factorial PBD trials were carried out in order to select the important factors that influence decolourisation of remazol golden yellow by *M. endophyticus* ES37 and also to evaluate the relative importance of the significant factors that are involved in the process. Scrutinised factors and their actual values are shown in Table 1. Plackett–Burman experimental design, depicted in Table 2, is based on the first-order model Equation (2) as follows:

$$Y = \beta_0 + \Sigma\beta_{\mathrm{i}}X_i + \Sigma\beta_{\mathrm{ii}}X_i^2 + \Sigma\beta_{\mathrm{ij}}X_iX_i \tag{2}$$

where Y is the response, X_i is factor levels, i is the factor number, β_0 is the model intercepts term, β_{i} is the linear effect, β_{ii} is the squared effect, and β_{ij} is the interaction effect between X_i and X_j on the dye decolourisation process.

A Plackett–Burman experimental run was performed in a basal broth medium with abiotic controls in static incubation. Response percentage dye decolourisation was assayed. A

Table 1 | Actual values of factors for PBD

Test variable	Lactose % (w/v)	Yeast extract % (w/v)	pH	Temperature (°C)	Inoculums size % (v/v)	Dye concentration (mg/L)	Incubation period (h)
Low level (−)	0.1	0.1	5	30	5	100	24
High level (+)	1.0	1.0	9	45	10	300	72

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Table 2 | Plackett–Burman experimental design for screening of significant factors on dye decolourisation

Run order	Lactose % (w/v)	Yeast extract % (w/v)	pH	Temperature (°C)	Inoculum size % (v/v)	Dye concentration (mg/L)	Incubation period (h)	DV-1*	DV-2*
1	1.0	0.1	9	30	5	100	72	1	1
2	1.0	1.0	5	45	5	100	24	1	1
3	0.1	1.0	9	30	10	100	24	−1	1
4	1.0	0.1	9	45	5	300	24	−1	−1
5	1.0	1.0	5	45	10	100	72	−1	−1
6	1.0	1.0	9	30	10	300	24	1	−1
7	0.1	1.0	9	45	5	300	72	−1	1
8	0.1	0.1	9	45	10	100	72	1	−1
9	0.1	0.1	5	45	10	300	24	1	1
10	1.0	0.1	5	30	10	300	72	−1	1
11	0.1	1.0	5	30	5	300	72	1	−1
12	0.1	0.1	5	30	5	100	24	−1	−1

*DV 1 and 2, dummy variable; +1, high concentration; −1, low concentration.

positive or negative coefficient value of factors specifies their relative contribution to dye decolourisation (Du *et al.* 2012). A larger magnitude of Student *t*-test value with smaller *P*-value of factors indicates higher significance on dye decolourisation (Karthikeyan *et al.* 2010). Only the factors which had a *P*-value (probability > F) of less than 0.10 were considered for further experiments (Khelifi *et al.* 2012).

RSM

The significant factors involved in decolourisation were optimised using RSM and central composite design (CCD). RSM and CCD involve a group of trial systems employed to assess the correlation between experimental factors and the response. The selected factors, namely yeast extract, lactose and pH, were considered at five coded levels, $-\alpha$, -1, 0, +1 and $+\alpha$ (Table 3); $\alpha = 2^{n/3}$, 'n' is the number of parameters and '0' is the central point. The actual values of experimental factors were calculated by using Equation (3) (Paul *et al.* 1992):

$$\text{Coded value} = \frac{\text{Actual value} - (\text{high level} + \text{low level})/2}{(\text{High level} - \text{low level})/2} \quad (3)$$

Table 3 | Actual values of the significant factors

Variables	Unit	Five levels of variables −α (−1.68179)	−1	0	1	+α (+1.68179)
Yeast extract	% (w/v)	– 0.20681	0.1	0.55	1	1.306807
Lactose	% (w/v)	– 0.20681	0.1	0.55	1	1.306807
pH	–	3.636414	5	7	9	10.36359

The experimental model of CCD is shown in Table 4. All the other factors used in this study were constant: 0.5% (w/v) sodium chloride, 200 mg/L dye concentration, 18 h culture (OD 610 nm = 0.5) of 7.5% (v/v) inocula, 37 °C temperature and 48 h incubation period. The correlation and relationship of these factors and their measured response in CCD can be described by linear quadratic model as in Equation (4):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{32} + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (4)$$

where Y is the percentage of decolourisation, β_0 is the intercept term, β_1, β_2 and β_3 are linear coefficients of each significant factor, β_{11}, β_{22}, β_{33} are quadratic coefficients, β_{12}, β_{13}, β_{23} are interaction coefficients and X_1, X_2, X_3, X_4 are coded factors. The best experimental model was determined

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Table 4 | CCD matrix for optimisation of dye decolourisation

Run order	Pt type	Blocks	Yeast extract % (w/v)	Lactose % (w/v)	pH
1	1	1	0.1	0.1	5
2	1	1	1.0	0.1	5
3	1	1	0.1	1.0	5
4	1	1	1.0	1.0	5
5	1	1	0.1	0.1	9
6	1	1	1.0	0.1	9
7	1	1	0.1	1.0	9
8	1	1	1.0	1.0	9
9	−1	1	−0.20	0.55	7
10	−1	1	1.31	0.55	7
11	−1	1	0.55	−0.20	7
12	−1	1	0.55	1.31	7
13	−1	1	0.55	0.55	3.63
14	−1	1	0.55	0.55	10.36
15	0	1	0.55	0.55	7
16	0	1	0.55	0.55	7
17	0	1	0.55	0.55	7
18	0	1	0.55	0.55	7
19	0	1	0.55	0.55	7
20	0	1	0.55	0.55	7

by coefficient R^2. A regression model equation was used to find the predicted optimal values of the selected factor and to subsequently reveal the interaction of these factors within a stipulated level (Zhao *et al.* 2010). The maximum decolourisation potential of the bacteria was verified under predicted optimal condition, obtained in RSM. Experiments were conducted in triplicate and the decolourisation percentage was compared to the predicted result from RSM.

RESULTS AND DISCUSSION

Enumeration and screening of dye-decolourising bacteria

The total heterotrophic bacterial population of the textile dye contaminated soil was found to be 62×10^6 CFU/g. The presence of a high bacterial population in the dye-contaminated soil has drawn attention to the utilisation of native azo dye resistant bacteria in dye degradation (Rajee & Patterson 2011). Dye decolourisation efficiency of distinct bacterial colonies using plate assay at different concentrations, ranging from 50 to 250 mg/L of remazol golden yellow dye in a Luria-Bertani medium, was determined. Twenty bacterial colonies

Table 5 | PBD of experimental and predicted decolourisation (%)

Run order	Lactose % (w/v)	Yeast extract % (w/v)	pH	Temperature (°C)	Inoculum size % (v/v)	Dye concentration (mg/L)	Incubation period (h)	Percentage decolourisation	
								Experimental	Predicted
1	1.0	0.1	9	30	5	100	72	6.91	7.023
2	1.0	1.0	5	45	5	100	24	7.28	7.89
3	0.1	1.0	9	30	10	100	24	21.55	18.08
4	1.0	0.1	9	45	5	300	24	4.88	3.7083
5	1.0	1.0	5	45	10	100	72	8.17	7.56
6	1.0	1.0	9	30	10	300	24	7.65	11.11
7	0.1	1.0	9	45	5	300	72	22.57	20.56
8	0.1	0.1	9	45	10	100	72	7.38	10.34
9	0.1	0.1	5	45	10	300	24	1.64	1.79
10	1.0	0.1	5	30	10	300	72	0.63	−1.85
11	0.1	1.0	5	30	5	300	72	13.34	15.32
12	0.1	0.1	5	30	5	100	24	5.08	5.43

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were observed to possess a positive zone of clearance at 100 mg/L concentration. The ratio of removal of dye by bacteria was directly propotional to the concentration of the dye (Jadhav *et al.* 2008). *M. endophyticus* ES37 produced clear zones in all the concentrations of dye and was scrutinised for further investigations. Elisangela *et al.* (2009) have emphasised that dye-decolourising micro-organisms from contaminated samples of the textile industry have maximum dye removal capacity when compared to the strains that are isolated from other sources.

Identification of dye-decolourising bacteria

The identification of the dye-decolourising bacterial strain was done by 16S rDNA sequencing method. The evolutionary history was analysed using the NJ method. The phylogenetic tree of the sequenced strain is shown in Figure 1. The tree was constructed from nucleotide sequences of ES37, which demonstrated 99% similarity with *M. endophyticus* YIM 56238 (accession number EU005372). Hence, the strain was identified as *Micrococcus endophyticus* ES37 (accession number JX042488). A number of *Micrococcus* sp. has been reported for decolourisation of textile dyes in the previous literature; however, no reports are available on decolourisation of dyes using *M. endophyticus*. Similar to *M. endophyticus*, the strains *Micrococcus luteus* (accession number AJ409096) and *Micrococcus* sp. (accession number AJ313024) also have the ability to decolourise the dye present in contaminated soil (Moosvi *et al.* 2007).

Decolourisation of remazol golden yellow in various compositions

Growth of micro-organisms mainly depends upon the supplementation of nutrients in media. Nutrients are necessary for microbial growth and cell metabolic activities (Moosvi *et al.* 2007). The decolourisation ability of *M. endophyticus* ES37 was observed using different media compositions. The strain showed 53.59% dye removal within 24 h. After additional incubation for 48 h, the bacterial cells achieved

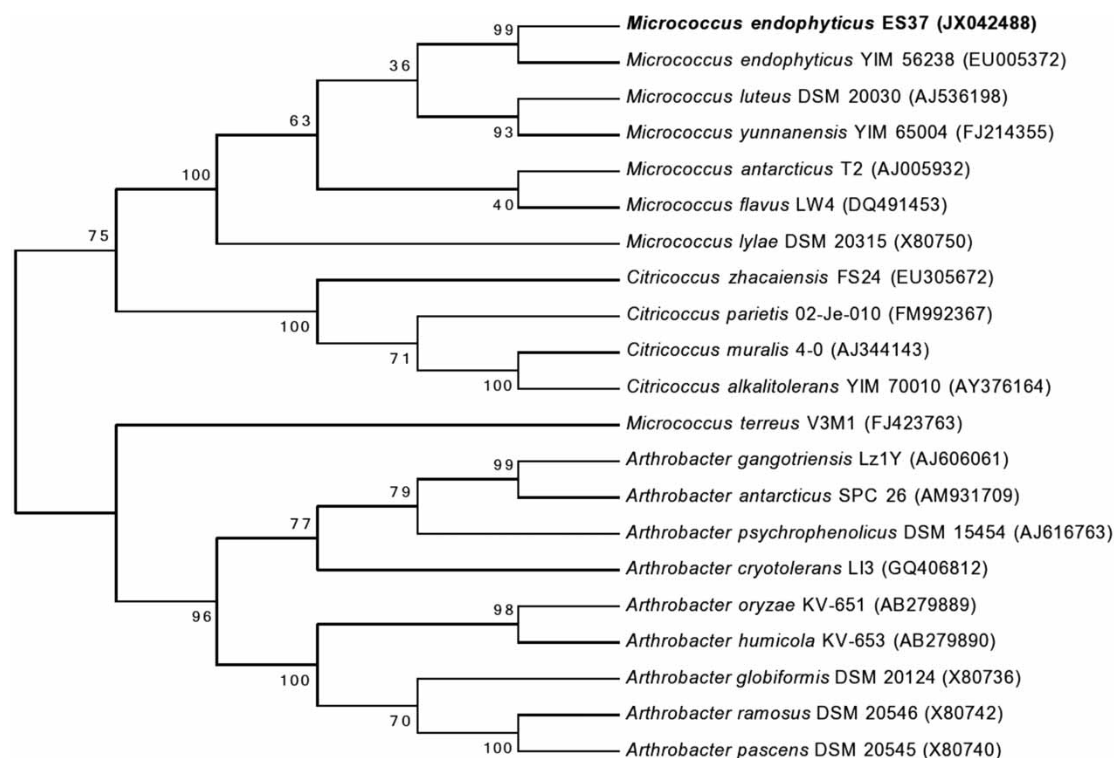

Figure 1 | Phylogenetic tree of *Micrococcus endophyticus* (ES37) showing the relationship between selected bacterial strains. The per cent numbers at the nodes indicate the levels of bootstrap support based on NJ analyses of 1,000 replicates. Brackets represents sequence accession numbers.

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97.19% decolourisation in Luria-Bertani broth composition. Saratale *et al.* (2010) reported that *Micrococcus glutamicus* NCIM-2168 required 42 h in order to achieve maximum decolourisation of dyes, which may be due to the higher molecular weight and presence of inhibitory groups in the dyes. The maximum decolourisation ratio in LB broth was enhanced by amendment of casein enzymic hydrolysate. In yeast extract broth, the culture exhibited 6.52% and 15.23% decolourisation after the incubation period of 24 h and 48 h, respectively. Finally, the strain achieved 53.4% decolourisation of remazol golden yellow dye in 72 h. Similar results were observed in the decolourisation of Orange MR dye at 900 ppm by *Micrococcus* sp. DBS 2; decolourisation of up to 36.34% has been reported (Rajee & Patterson 2011). The organic nitrogen source, contributed by the yeast extract in the media, is used to reduce azo bonds by regeneration of NADH as an electron donor (Chen *et al.* 2003). However, the extended incubation period of *M. endophyticus* ES37 in Bushnell Haas broth composition failed to decolourise remazol golden yellow dye after using it as the sole source of carbon and nitrogen. The absence of supplementary carbon and nitrogen sources in the bacterial culture exemplifies the essentiality of a co-substrate to support bacterial growth and decolourisation (Nigam *et al.* 1996).

Optimisation of dye decolourisation

Screening of significant factors by PBD

The influence of obligatory factors under static conditions, during decolourisation of remazol golden yellow by *M. endophyticus* ES37, was analysed using PBD. There was a wide variation in efficiency of remazol golden yellow decolourisation, ranging from 0.63 to 22.57% (Table 5). The statistical analyses of regression coefficients and estimated effects of the factors are given in Table 6. The coefficient values of yeast extract, pH and incubation period indicates that the increase in their concentration has a positive influence on the decolourisation process. Conversely, lower temperature, lactose concentration, inocula size and dye concentration are required to assist maximum dye removal ratio. Du *et al.* (2012) reported that yeast extract and pH had a positive effect on decolourisation of malachite green by *Micrococcus* sp. strain BD15, whereas a higher concentration of dye and higher temperature had negative effects on decolourisation; this supports the present observation. Anjaneya *et al.* (2011) pointed out that higher temperature leads to the loss of microbial metabolic activities during dye decolourisation. The necessity of using a lower concentration of dye and lactose in the culture medium may be related to the toxic effect on the bacterial cells, inhibition of azo reductase active sites and other enzyme systems involved in decolourisation (Tony *et al.* 2009; Jadhav *et al.* 2010). In the present study, factors possessing confidence levels greater than 90% were considered significant. A large *t*-value coupled with a low *P*-value specifies high significance of factors and model term.

The effect of significant factors on decolourisation is explained by regression Equation (5), which is used to find the predicted decolourisation (%) of any desired combination of factors in the experimental range. The fittest model was confirmed by the determination of correlation

Table 6 | Estimated effects of PBD

S. no.	Variable	Effect	Coef.	SE coef.	*T*	*P*
1	Constant	–	8.916	1.011	8.82	0.001*
2	Lactose	−6.018	−3.009	1.011	−2.98	0.041*
3	Yeast extract	9.015	4.507	1.011	4.46	0.011*
4	pH	5.782	2.891	1.011	2.86	0.046*
5	Temperature	−0.545	−0.272	1.011	−0.27	0.801
6	Inoculum size	−2.152	−1.076	1.011	−1.06	0.347
7	Dye concentration	−0.948	−0.474	1.011	−0.47	0.663
8	Incubation period	1.822	0.911	1.011	0.90	0.418

*Significant.
$R^2 = 90.74\%$ $R^2(\text{adj}) = 74.53\%$.

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coefficient ($R^2 = 0.9074$), which is nearer to 1; it is denoted for good statistical replication between the experimental and predicted responses.

$$Y = 8.916 - 3.009 \times \text{lactose} + 4.507 \times \text{yeast extract} + 2.891 \times \text{pH} - 0.272 \times \text{temperature} - 1.076 \times \text{inoculum size} - 0.474 \times \text{dye concentration} + 0.911 \times \text{incubation period} \quad (5)$$

Analysis of variance (ANOVA) of the linear model is presented in Table 7. This explains the presence of variations in the net result caused by each factor that is relative to the total variation. According to a Pareto chart (Figure 2) the screened factors, in the order yeast extract, lactose and pH, were most effective for remazol golden yellow dye decolourisation.

RSM approach using CCD

The significant factors (yeast extract, lactose and pH) and their combined effect were studied by RSM for optimisation of remazol golden yellow decolourisation using *M. endophyticus* ES37. As per CCD, the dye removal rate varied from 0.43 to 77.49% (Table 8). Second-order polynomial model Equation (6) and CCD results were linked in order to elucidate the dependence of the dye removal ratio by multiple regression analysis. The estimated regression coefficient for the model is given in Table 9. The magnitude of coefficient had high significance ($P = 0$) for all factors during the decolourisation process. In addition, the correlation coefficient (R^2) was determined by regression analysis and was found to be 83.43%, thus a higher value of R^2 indicated a good correlation between the factors and CCD dye decolourisation process.

$$Y = 72.5917 + 16.3401 \times X_1 + 5.6242 \times X_2 + 8.6070 \times X_3 - 11.1646 \times X_1^2 - 6.1866 \times X_2^2 - 13.0031 \times X_3^2 - 2.1575 \times X_1 \times X_2 - 3.4575 \times X_1 \times X_3 + 0.0725 \times X_2 \times X_3 \quad (6)$$

where Y is predicted dye decolourisation (%), and X_1, X_2 and X_3 were the coded values of lactose, yeast extract and pH, respectively.

ANOVA was used to evaluate the implication and competence of the fittest second-order polynomial model on maximum dye decolourisation. Linear ($P = 0.003$) and quadratic ($P = 0.007$) effects of the factors had a greater influence on the dye decolourisation process. Nevertheless, the interaction terms ($P = 0.867$) were not statistically significant. This indicates that all the factors had a specific effect on decolourisation. Counter plots of a range of responses with the desired combination of significant factors in the experimental range are illustrated in Figure 3. These figures signify the relationship and interaction effect of any two factors with varying concentrations tested on dye decolourisation, while the third factor was maintained in the mid-value.

Table 7 | ANOVA for PBD

S. No.	Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
1	Main effects	7	480.188	480.188	68.598	5.60	0.058*
2	Lactose	1	108.661	108.661	108.661	8.87	0.041*
3	Yeast extract	1	243.811	243.811	243.811	19.90	0.011*
4	pH	1	100.283	100.283	100.283	8.18	0.046*
5	Temperature	1	0.891	0.891	0.891	0.07	0.801
6	Inoculum size	1	13.889	13.889	13.889	1.13	0.347
7	Dye concentration	1	2.698	2.698	2.698	0.22	0.663
8	Incubation period	1	9.955	9.955	9.955	0.81	0.418
9	Residual error	4	49.015	49.015	12.254	–	–
	Total	11	529.204				

*Significant.

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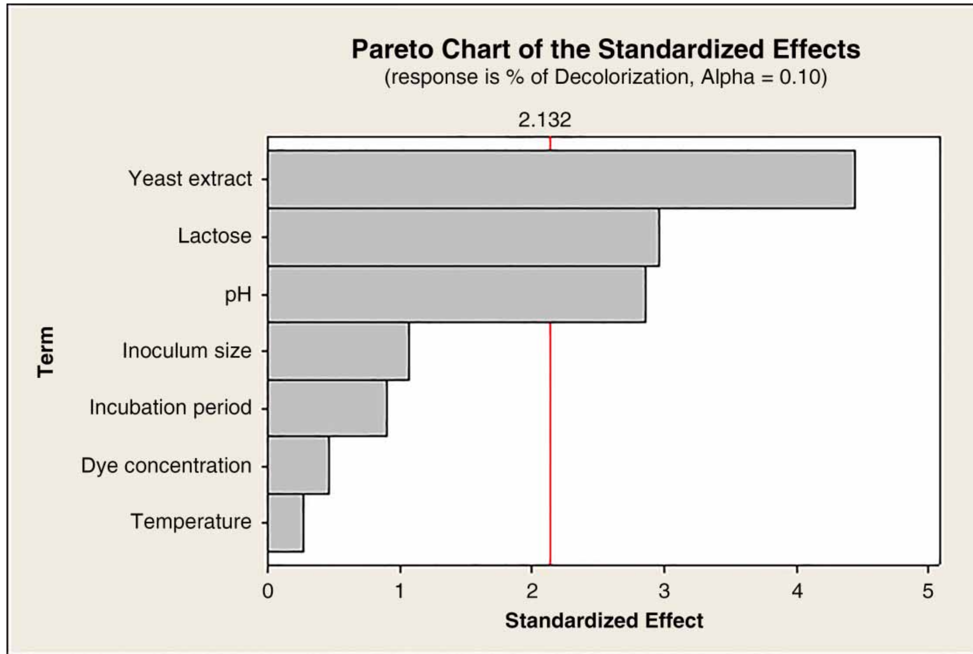

Figure 2 | Effects of influencing factors on dye decolourisation in a Pareto chart.

Table 8 | CCD mediated decolourisation (%) with predicted values

Trial	Lactose (X_1)	Yeast extract (X_2)	pH (X_3)	Percentage decolourisation		Residual
				Experimental	Predicted	
1	0.1	0.1	5	22.09	6.12	15.96
2	1	0.1	5	56.68	50.03	6.64
3	0.1	1	5	31.55	21.54	10.00
4	1	1	5	60.2	56.82	3.37
5	0.1	0.1	9	27.76	30.10	−2.34
6	1	0.1	9	51.21	60.18	−8.97
7	0.1	1	9	40.2	45.81	−5.61
8	1	1	9	52.33	67.26	−14.93
9	−0.20	0.55	7	3.32	13.53	−10.21
10	1.31	0.55	7	77.25	68.49	8.75
11	0.55	−0.20	7	39.42	45.63	−6.21
12	0.55	1.31	7	69.31	64.55	4.75
13	0.55	0.55	3.64	0.43	21.33	−20.90
14	0.55	0.55	10.36	69.74	50.28	19.45
15	0.55	0.55	7	74.81	72.59	2.21
16	0.55	0.55	7	77.49	72.59	4.89
17	0.55	0.55	7	71.3	72.59	−1.29
18	0.55	0.55	7	70.99	72.59	−1.60
19	0.55	0.55	7	70.5	72.59	−2.09
20	0.55	0.55	7	70.71	72.59	−1.88

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Table 9 | Estimated regression coefficients for CCD

S. no.	Variable	Coef.	SE coef.	T	P
1	Constant	72.5917	5.548	13.085	0.000*
2	Lactose	16.3401	3.681	4.439	0.001*
3	Yeast extract	5.6242	3.681	1.528	0.158
4	pH	8.6070	3.681	2.338	0.041*
5	Lactose*Lactose	−11.1646	3.583	−3.116	0.011*
6	Yeast extract*Yeast extract	−6.1866	3.583	−1.727	0.115
7	pH*pH	−13.0031	3.583	−3.629	0.005*
8	Lactose*Yeast extract	−2.1575	4.809	−0.449	0.663
9	Lactose*pH	−3.4575	4.809	−0.719	0.489
10	Yeast extract*pH	0.0725	4.809	0.015	0.988

*Significant.
$R^2 = 83.43\%$ R^2(adj) $= 68.51\%$.

In the validation study, a response optimiser was applied to establish optimal concentrations of important factors: lactose, yeast extract and pH were found to be 0.85% (w/v), 0.71% (w/v), and 7.5%, respectively. Under these predicted optimal conditions, about 81.61% of decolourisation was attained in accordance with a predicted value of 79.99%. Based on the results observed by Saratale *et al.* (2009), added nutrients are substrates needed for the functioning of the azo reductase enzyme system. Rajee & Patterson (2011) observed that optimal pH 6 is required for decolourisation of azo dye by an autochthonous bacterium: *Micrococcus* sp. DBS; pH contributes to the transportation of dye molecules across the cell membrane. Similar to the present study, Du *et al.* (2012) reported that under optimised conditions, decolourisation of malachite green by *Micrococcus* sp. strain BD15 was achieved with an efficiency of 96.9%, which was within the predicted level (92.04%). *M. glutamicus* NCIM 2168 completely decolourised an initial concentration of 50 mg/L sulphonated diazo dye Reactive Green 19A within 42 h at 37 °C and pH 8 (Saratale *et al.* 2009). Under optimal conditions, decolourisation of textile wastewater increased significantly; this may be due to the influence of variations in abiotic factors that are involved in the decolourisation process (Chaudhari *et al.* 2013).

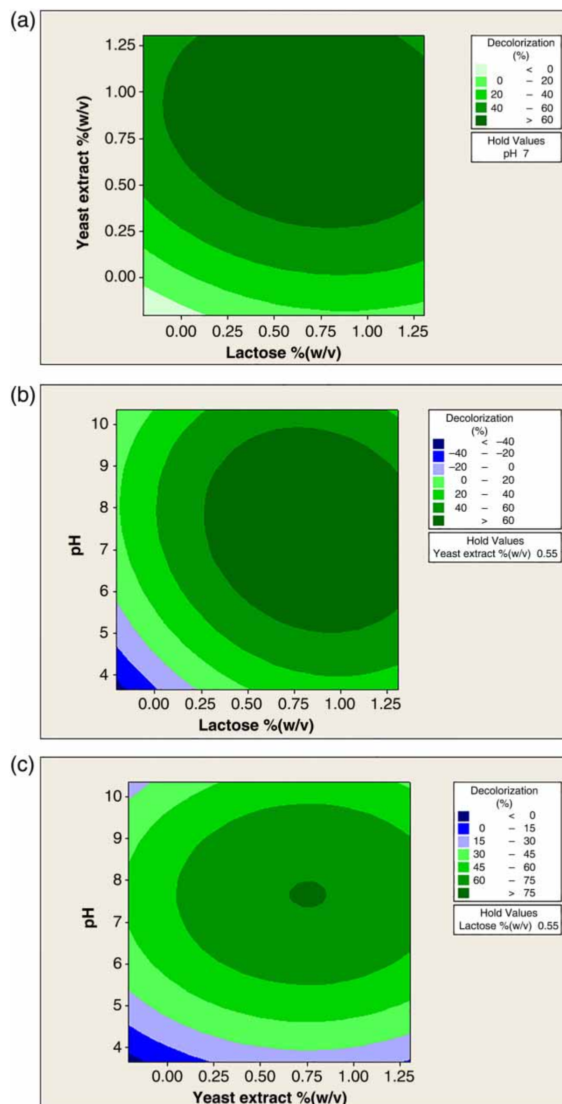

Figure 3 | Response contour plots between significant factors on decolourisation (%): (a) effect of yeast extract and lactose concentration on dye decolourisation (%); (b) effect of pH and lactose concentration on dye decolourisation (%); (c) effect of pH and yeast extract concentration on dye decolourisation (%).

CONCLUSION

The present study clearly emphasises that the indigenous bacterial strain *M. endophyticus* ES37 has the ability for bioremediation of azo dye. The interactive effect of significant parameters and their optimal conditions were determined by statistical applications. The favourable conditions of environmental parameters most suitable for dye decolourisation resembled normal conditions. However,

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the toxicity of the degraded substances must be analysed prior to industrial applications.

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